# Exposure-Disease Continuum for 2-Chloro-2′-Deoxyadenosine, a Prototype Ocular Teratogen. 3. Intervention with PK11195

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Received 12 August 2002; Revised 18 November 2002; Accepted 18 November 2002

**BACKGROUND:** Treatment of pregnant mice with 2-chloro-2′-deoxyadenosine (2CdA) on Day 8 of gestation induces microphthalmia through a mechanism linked to the p53 tumor suppressor pathway. The present study defines the response of Day 8 mouse embryos through time with respect to pharmacologic intervention with PK11195, a ligand of the mitochondrial peripheral benzodiazepine receptor (Bzrp). **METHODS:** Pregnant CD-1 mice dosed with 2CdA with or without PK11195 on gestation Day 8 provided fetuses for teratologic evaluation on Day 14 and Day 17; HPLC measured pyridine nucleotides ($NADH/NAD^+$) at 1.5 hr, RT-PCR measured mitochondrial 16S rRNA abundance at 3.0 hr, and p53 protein induction was assessed with immunostaining at 4.5 hr postexposure. **RESULTS:** The mean incidences of malformed fetuses were significantly higher in the 7.5 mg/kg 2CdA treatment group (50.2% malformed) vs. the 2CdA + 4.0 mg/kg PK11195 co-treatment group (4.4% malformed). Malformed fetuses displayed a range of ocular defects that included microphthalmia and keratolenticular dysgenesis (Peters anomaly). No malformations were observed in the control or PK11195 alone groups. PK11195 also protected litters from increased resorption rates and fetal weight reduction. It did not rescue early effects on NADH balance (1.5 hr) or 16S rRNA expression (3.0 hr); however, the p53 response (4.5 hr) was downgraded in 2CdA + PK11195 embryos vs. 2CdA alone. By delaying the administration of PK11195 in 1.5 hr intervals it was determined that the window for protection closed between 4.5 to 6.0 hr after 2CdA. **CONCLUSIONS:** The capacity of PK11195 to suppress the pathogenesis of microphthalmia implies a critical role for mitochondrial peripheral benzodiazepine receptors in the p53-dependent mode of action of 2CdA on ocular development. *Birth Defects Research (Part A) 67:108–115, 2003.* © 2003 Wiley-Liss, Inc.

## INTRODUCTION

An important question in teratogenesis pertains to how adverse effects at the subcellular level cause specific developmental abnormalities and in particular when after exposure do the critical changes occur. The nucleoside analogue 2-chloro-2′-deoxyadenosine (2CdA) is a useful model agent for addressing this question. Its natural congener, 2′-deoxyadenosine (dA), is a metabolic toxin endemic to the early postimplantation uterus (Knudsen et al., 1992; Blackburn et al., 1997). Unlike the natural congener, however, 2CdA is metabolically stable and rapidly reaches the embryo after maternal dosing (Lau et al., 2002). Both dA and 2CdA may trigger excessive programmed cell death (apoptosis) in cranial neural folds while sparing the primitive heart (Gao et al., 1994; Wubah et al., 1996). The mechanism of action is unknown.

2CdA is an ocular teratogen in mouse and rat embryos exposed at the headfold stage of development (Wubah et al., 2001; Lau et al., 2002). Microphthalmia appeared first in the 2CdA dose–response curve for both species and was designated the critical effect malformation. The benchmark dose that produced an extra 5% risk of microphthalmia ($BMD_5$) differed for mouse and rat, however, with the former being more the sensitive species ($BMD_5$ = 2.5 mg/kg vs. 19.4 mg/kg, respectively). Susceptibility of mouse embryos to 2CdA is at least partly determined by the *Trp53* tumor suppressor gene because incidences of micro-/anophthalmia segregated with the wild-type allele vs. the *Trp53*(Δ5) null mutant allele (Wubah et al., 1996).

Knowledge about the potential mechanism of action of 2CdA has largely come from studies focused on its unique cytotoxicity toward slow-growing leukemia cells. One proposed mechanism is differential bioactivation to 2CdAMP mediated either by deoxycytidine kinase (dCK) in the nucleus or deoxyguanosine kinase (dGK) in the mitochondrion (Wang et al., 1993, 1996; Johansson et al., 1997; Zhu et al., 1998; Jullig and Eriksson, 2000). The mitochondrial pathway is the most likely route for 2CdA bioactivation in early mouse embryos although differential bioactivation does not explain the tissue differences in cytotoxicity be-

Grant sponsor: US Environmental Protection Agency; Grant number: STAR grant R82744501-01; Grant sponsor: National Institute of Environmental Health Sciences; Grant numbers: RO1 ES09120, T32 ES07282.
The views expressed herein are solely the responsibility of the authors and do not necessarily represent the official views or policies of the NIEHS or the EPA.
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Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bdra.10026

tween the embryonic headfold and heart (Wubah et al., 2001). A second proposed mechanism of action of 2CdA pertains to its misincorporation via 2CdATP into mitochondrial DNA (mtDNA) and the consequences to mtDNA replication and cellular bioenergetics (Chunduru et al., 1993; Hentosh and Grippo, 1994; Hentosh and Tiduban, 1997). Preliminary analysis of high-energy nucleotides in early mouse embryos revealed perturbation of cellular energy balance 1.5 hr postexposure reflected in a lower ratio of ATP/ADP or $NADH/NAD^+$. Furthermore, mitochondrial 16S rRNA transcripts were significantly elevated at 3.0 hr postexposure (Wubah, 1998). These preliminary findings suggest that sensitive embryonic cells undergo a metabolic reprogramming in response to 2CdA exposure, a response that supports the notion of a mitochondrial mechanism of action. A third proposed mechanism of action of 2CdA pertains to stimulation of apoptosis through decreases in mitochondrial transmembrane potential, release of cytochrome c from the mitochondrion, and Apaf-1-mediated caspase activation (Leoni et al., 1998; Chandra et al., 2002). Regulation of these events may be linked with p53 protein induction because nuclear p53-immunoreactivity increased in cranial neural folds between 3.0 and 4.5 hr after 2CdA exposure but not the heart; furthermore, teratogenesis correlated directly with p53 protein induction, p53-dependent apoptosis, and the embryo's genotype with respect to the *Trp53* allele (Wubah et al., 1996, 2001).

To better understand how p53 couples teratogen (2CdA) exposure with disease (microphthalmia), and in particular when during exposure the steps leading to malformation become irreversible, the present study has investigated the temporal changes leading to p53 protein induction in the headfold of Day 8 mouse embryos utilizing isoquinoline carboxamide derivative PK11195. This small molecule influences mitochondrial function via high-affinity binding to the mitochondrial peripheral benzodiazepine receptor (Bzrp) (see Casellas et al., 2002). It significantly lowers the risk of oculocerebral malformations in mouse fetuses exposed to methylmercury during early organogenesis (O'Hara et al., 2002) and is shown in the present study to substantially reduce the risk for microphthalmia in early mouse embryos exposed to 2CdA.

## MATERIALS AND METHODS

### Materials

2CdA was a gift from Dr. James Oldham of R.W. Johnson Pharmaceutical Research Institute (Spring House, PA). Standard biochemicals purchased from Fisher Scientific (Fairlawn, NJ) or Sigma Chemical Company (St. Louis, MO) were of the highest grade available. 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) and 2-hydroxypropyl-$\beta$-cyclodextrin (HP-beta-CD) were from Sigma-RBI. Sheep polyclonal antiserum Ab-7 to recombinant human p53 was purchased from Oncogene Research Products (Cambridge, MA). SK-4100 DAB peroxidase substrate was purchased from Vector Laboratories, Inc., (Burlingame, CA). RNeasy microspin kits were from QIAGEN, Inc., (Valencia, CA). Superscript II RT, random primers (HAS701), RNase-free DNase I and other reverse-transcriptase PCR (RT-PCR) reagents were from Gibco-BRL (Gaithersburg, MD). Ready-to-go PCR beads were from Amersham (Piscataway, NJ). Water for solutions and buffers was collected at 18.2 M$\Omega$-cm from the Milli-Q Plus Ultra-pure water system from the Millipore Corporation (Milford, MA).

### Animals and Treatments

The Institutional Animal Use and Care Committee at Thomas Jefferson University approved all protocols describing the animal research reported here. Outbred CD-1 mice (20–30 gm) were purchased from Charles River Breeding Laboratories (Wilmington, MA), housed on a 12-hr photoperiod (07:00–19:00 hr light), and fed Purina mouse chow and water *ad libitum*. Timed pregnancies were generated by caging a male with nulliparous females at 07:30 hr. Detection of a vaginal plug at 12:30 hr signified coitus (gestational Day 0). Dams were euthanized by carbon dioxide for HPLC analysis (1.5 hr), RT-PCR (3.0 hr), p53 immunohistochemistry (4.5 hr), ocular histology (Day 14), and teratological evaluation (Day 17).

Gravimetric 2CdA stock solutions were calibrated by absorbance at 264 nm using 15.0 as the millimolar extinction coefficient (Liliemark, 1997). Stock solutions diluted with sterile water were prepared to deliver the prescribed dose when injected in 0.2 ml/0.03 kg of maternal body weight. All 2CdA stock and dosing solutions were stored frozen (−20°C) in single-use aliquots. Pregnant mice received 2CdA by a single intraperitoneal (i.p.) injection at 09:30 hr on Day 8. Historically, most CD-1 mouse embryos at this time have 4–6 somite pairs (Wubah, 1998). Dosages of 2CdA were 5.0 mg/kg or 7.5 mg/kg, as indicated. For co-treatment with PK11195, the lipophilic drug was prepared in 4.5% HP-beta-CD in water followed by extensive mixing and brief sonication at room temperature to yield a 11:1 molar ratio of inclusion compound and PK11195 (O'Hara et al., 2002). HP-beta-CD is a non-toxic solubilizer that dissolves drugs through the formation of inclusion complexes (Pitha and Pitha, 1985). The solution was injected i.p. to deliver a dose of 4.0 mg/kg PK11195 (0.12 ml/0.03 kg body weight) either immediately before 2CdA (<30 sec, co-treatment) or at various times after 2CdA (1.5–6.0 hr).

### Teratological Evaluation

In the first round of experiments, pregnant CD-1 dams were assigned to four treatment groups: vehicle control (water), 2CdA (7.5 mg/kg in water), PK11195 (4.0 mg/kg in HP-beta-CD), or 2CdA with PK11195 (co-treatment). The 2CdA dose represents a strong embryotoxic effect that includes ocular malformations, increased resorptions, and fetal weight reduction (Wubah et al., 2001). The PK11195 dose represents one that is pharmacologically active in rodents (e.g. 5.0 mg/kg) (Wala et al., 2000) and one that can partially abrogate the teratogenicity of methylmercury in CD-1 mice (O'Hara et al., 2002). Each experimental group consisted of seven litters. A second round of experiments was conducted with delayed PK11195 administration. In these experiments, dams received a moderately teratogenic dose of 5.0 mg/kg 2CdA followed with 4.0 mg/kg PK11195 given 1.5 hr, 3.0 hr, 4.5 hr, or 6.0 hr after 2CdA. Each delayed co-treatment group had three to six litters. After recording implantations and resorptions, fetuses were examined for gross malformations, weighed, euthanized, and fixed in formalin to cloud the lens. Eyes were inspected for gross structural change to the optic globe under a widefield stereoscope. An eye was classified as

microphthalmic if the lens was visibly smaller than normal and anophthalmic when extremely reduced or absent. To grade eye reduction defects (ERDs), a value was assigned to each eye: 0 if normophthalmic, 2 if microphthalmia, and 3 if anophthalmic; the cumulative value for the entire population was then divided by the number of eyes (Wubah et al., 2001). One-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison post test was carried out using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA).

### p53-Immunostaining

Embryo-decidual units were fixed in neutral-buffered formalin overnight at 4°C and embedded in paraffin (Wubah et al., 2001). Semi-serial sections were cut at 5-microns and collected on positively charged Superfrost/plus microscope slides. Deparaffinated sections were rehydrated and subjected to microwave retrieval at 95–100°C for 10 min in 0.01M sodium citrate, pH 6.0. Sections were rinsed in phosphate-buffered saline (PBS) and subjected to peroxidase block. Sheep polyclonal antiserum Ab-7 was applied at a dilution of 1:500 in 0.5% BSA-PBS. Incubation was overnight at 4°C. Normal sheep serum provided the negative control. Primary antiserum was localized by sequential incubation with biotinylated rabbit anti-sheep serum (1:10,000 PBS) and streptavidin-OR03L-HRP peroxidase (1:200 PBS). Color development utilized DAB peroxidase substrate.

### Reverse Transcriptase PCR

Samples of the cranial neural fold (headfold) were harvested by microdissection from embryos on Day 8 of gestation (6–8 pooled per sample). RNA was extracted using RNeasy (QIAGEN). After 1 hr RNase-free DNase digestion 1 to 2 $\mu$g RNA was reverse transcribed with Superscript II RT at 42°C in the presence of dithiothreitol, random primers, and 0.2 mM each dNTP. Negative controls replaced reverse transcriptase with an equal volume of DEPC-treated water. Polymerase chain reaction (PCR) used Ready-to-go PCR beads, 2-$\mu$l of each sample cDNA, and 2-$\mu$l each primer in 25 $\mu$l volume. PCR cycles were 95°C (1 min), 66°C (2 min), and 72°C (1 min). Reference gene ($\beta$-actin) and target gene (16S rRNA) reactions used 24 cycles, which was optimal for a linear response under the conditions used for this study. The primers were: 5′-TAC CAC AGG CAT TGT GAT GG-3′ (upper) and 5′-AAT AGT GAT GAC CTG GCC GT-3′ (lower) for $\beta$-actin; 5′-ACA GCT AGA AAC CCC GAA AC-3′ (upper) and 5′-AAG ATA AGA GAC AGT TGG AC-3′ (lower) for 16S rRNA (O'Hara et al., 2002). PCR products were electrophoresed on 8% polyacrylamide gels with a 100 base pair DNA ladder as the marker. Gels were stained with ethidium bromide, photographed with a Kodak DC40 camera, and analyzed using Kodak's 1D photo imaging software. Each group had at least six independent samples ($n \geq 6$). Data analysis used one-way analysis of variance (ANOVA) and post analysis with Dunnett's multiple comparison test using GraphPad Prism.

### NADH Analysis

Day 8 embryos were extracted with 60% methanol at −20°C and the supernatant was dried in an Integrated Speedvac System (ISS110, Savant, Holbrook, NY). Samples were resuspended in mobile phase and analyzed by reversed-phase HPLC with ion-pairing. The method used a mobile phase of 50 mM ammonium phosphate, pH 6.5, with 5% acetonitrile and 2 mM tetra-butyl ammonium phosphate run at a flow rate of 1.5 ml/min (Gao et al., 1995). Samples were monitored with UV wavelength collection between 249 to 285 nm ($NAD^+$) and with in-line fluorescence excitation of 340 nm and emission of 465 nm (NADH). Standards were used in generating a calibration curve between 2 and 200 pmol for each component. Each group had at least five independent samples ($n \geq 5$). Data analysis used one-way analysis of variance (ANOVA) and post analysis with Dunnett's multiple comparison test.

## RESULTS

### PK11195 Co-Treatment Ameliorated 2CdA Developmental Toxicity

Pregnant mice on Day 8 of gestation received one of four exposures: vehicle control (water), 2CdA (7.5 mg/kg in water), PK11195 alone (4.0 mg/kg in HP-beta-CD), or 2CdA with PK11195 (co-treated). Table 1 gives the results of teratological evaluation on Day 17 of gestation ($n = 7$ litters per group). Group differences were evaluated using one-way ANOVA with Newman-Keuls multiple comparison post test. Significant differences were observed with respect to resorption rate, fetal weight, and malformation incidences. Higher resorption rates were observed in the 2CdA group (21%) vs. control, PK11195, or co-treated groups (<3%). Fetal weights were significantly lower in the 2CdA group (0.85 gm) vs. control, PK11195, or co-treated groups (> 0.99 gm). The mean incidences of malformed fetuses were significantly higher in the 2CdA group (50.2%) vs. the co-treated group (4.4%). Micro-/anophthalmia accounted for 30.5% eyes (36/118) and 2.1% eyes (3/142) in these two groups, respectively. No malformations were observed in the control (vehicle) or PK11195 alone groups. Therefore, PK11195 co-treatment rescued fetuses from microphthalmia and other adverse developmental outcomes caused by 2CdA exposure on gestation Day 8.

### Histological Confirmation in Day 14 Fetuses

Ocular malformations generally classified as eye reduction defects (ERDs) on the basis of gross changes to the ocular globe in term fetuses can range in severity from subtle narrowing of the papillary ring to complete apparent anophthalmia (Wubah et al., 1996, 2001). Because of the difficulty detecting subtle anatomical defects in a non-pigmented mouse strain we carried out histological analysis of several Day 14 fetuses to confirm the presence of structural abnormalities in the eyes of 2CdA exposed fetuses, as well as to confirm normal histological structure in the eyes of PK11195 co-treated fetuses. Microphthalmic eyes displayed alentia, microlentia, and cataract that are consistent with hypoplasia of the lens primordium; however, neotenous defects such as persistent hyaline vasculature and keratolenticular dysgenesis (Peters' anomaly) were also detected (Fig. 1). The latter malformation has been reported for exposure to common human teratogens such as ethanol or retinoic acid on Day 7–8 of gestation and is explained by failure of the lens to detach from the surface ectoderm, hence creating an obstacle for neural crest cells emigrating to the developing cornea or iris (Cook and Sulik, 1988). This histological change probably

Table 1
Developmental Toxicity of 2CdA Alone and in Combination with PK11195†

| Parameter | Control (vehicle) | 2CdA (7.5 mg/kg) | PK11195 (4.0 mg/kg) | Co-treated (2CdA + PK11195) | *p* (ANOVA) |
|---|---|---|---|---|---|
| Dams survived/treated, *n* | 7/7 | 7/7 | 7/7 | 7/7 | — |
| Implants per litter | 10.9 ± 2.9 (8.2–13.6) | 10.4 ± 2.0 (8.59–12.27) | 11.3 ± 1.9 (9.5–13.0) | 10.4 ± 2.4 (8.2–12.6) | 0.883 |
| Fetuses per litter | 10.4 ± 3.1 (7.6–13.3) | 8.4 ± 3.3 (5.42–11.44) | 11.0 ± 1.8 (9.3–12.7) | 10.1 ± 2.4 (7.9–12.4) | 0.338 |
| Resorptions, % | 2.6 ± 4.5 (0.0–6.8) | 20.9 ± 18.9▲▲ (3.40–38.40) | 2.4 ± 4.1 (0.0–6.21) | 2.7 ± 4.7▼▼ (0.0–7.05) | 0.0045* |
| Fetal weight in g | 1.024 ± 0.049 (0.979–1.069) | 0.850 ± 0.059▲▲ (0.796–0.904) | 1.068 ± 0.095 (0.980–1.155) | 0.994 ± 0.109▼▼ (0.893–1.095) | 0.0003* |
| Malformed fetuses, % | 0.0 ± 0.0 (0.0–0.0) | 50.2 ± 26.9▲▲▲ (25.3–75.0) | 0.0 ± 0.0 (0.0–0.0) | 4.4 ± 8.0▼▼▼ (0.0–11.9) | <0.0001* |
| Phenotype Normal/microphthalmia/anophthalmia (ERD grade) | 148/0/0 (0.000) | 82/16/20 (0.780) | 152/0/0 (0.000) | 139/3/0 (0.042) | — |

†Mean ± SD with exact 95% confidence interval (CI) in parenthesis. Statistical comparison by one-way ANOVA used unweighted litter means ($n = 7$); if ANOVA yielded a significant F-ratio at the designated alpha level ($p < 0.05$, *), then post-hoc analysis was performed with Newman-Keuls multiple comparison test versus normal (control, PK11195 ▲) or 2CdA alone (▼) groups for $p < 0.05$ (▲, ▼), $p < 0.01$ (▲▲, ▼▼), $p < 0.001$ (▲▲▲, ▼▼▼). Most malformed fetuses had micro-/anophthalmia; a small number had exencephaly without microphthalmia; phenotype refers to number of eyes scored for normophthalmia, microphthalmia, anophthalmia and overall eye reduction defects (ERD grade).

corresponds to narrowing of the papillary ring in term fetuses.

### Analysis of the Cellular p53 Response

Day 8 embryos were evaluated by p53 immunohistochemical staining at 4.5 hr after 5.0 mg/kg 2CdA with or without PK11195 co-treatment (Fig. 2). As expected, staining for p53 protein was uniformly weak in control (vehicle) embryos (Fig. 2A) in contrast to the positive response observed in cranial neural folds after 2CdA exposure (Fig. 2B). Omission of the primary antibody showed no residual staining in these exposed embryos (Fig. 2C). Furthermore, nuclear p53-immunoreactivity was substantially weaker in embryos co-treated with 2CdA with PK11195 (Fig. 2D) vs. 2CdA alone. This suggests that PK11195 co-treatment downgraded the effect that 2CdA exposure had on p53 protein induction that occurred by the 4.5 hr timepoint.

### NADH Balance and 16S rRNA Expression

Early changes in cellular mitochondrial function were confirmed by analysis of NADH levels reflected in an overall decreased ratio of NADH to $NAD^+$ at 1.5 hr postexposure, and the relative abundance of mitochondrial 16S rRNA transcripts reflected in an overall increased ratio of 16S rRNA to $\beta$-actin at 3.0 hr postexposure (Wubah, 1998). To determine if PK11195 intervention influenced these parameters, ratios of NADH (to $NAD^+$) and 16S rRNA (to $\beta$-actin) were determined for samples procured at 1.5 hr and 3.0 hr, respectively, after 5.0 mg/kg 2CdA alone or in combination with 4.0 mg/kg PK11195 (Fig. 3). Group differences were evaluated using one-way ANOVA with Dunnett's multiple comparison test post analysis. 2CdA exposure had significant effects on NADH levels and 16S rRNA abundance as reflected in a twofold drop in the

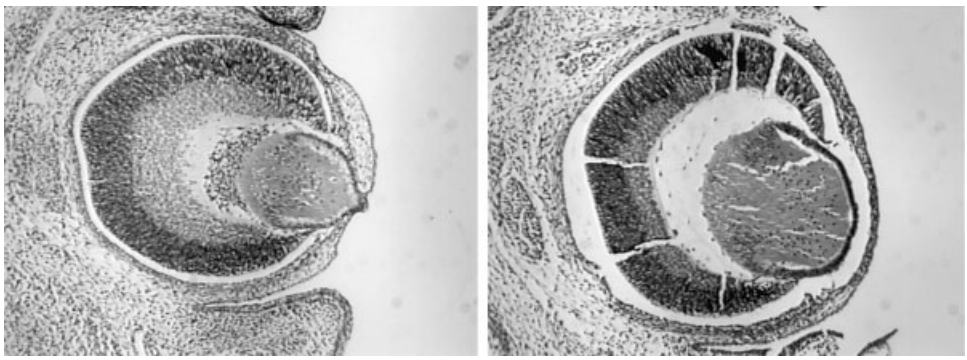

**Figure 1.** Keratolenticular dysgenesis (Peters' anomaly) in Day 14 mouse fetuses exposed to 2CdA on Day 8. Fetuses were formalin-fixed for hematoxylin and eosin staining. **Left:** Eye from microphthalmic fetus exposed to 7.5 mg/kg 2CdA on Day 8, displaying microlentia with vacuolation (cataract) and anterior herniation through the cornea, and persistent hyperplastic vitreous in the posterior chamber. **Right:** Eye from normophthalmic fetus exposed to 7.5 mg/kg 2CdA and co-treated with 4.0 mg/kg PK11195 on Day 8. Magnification = 48×.

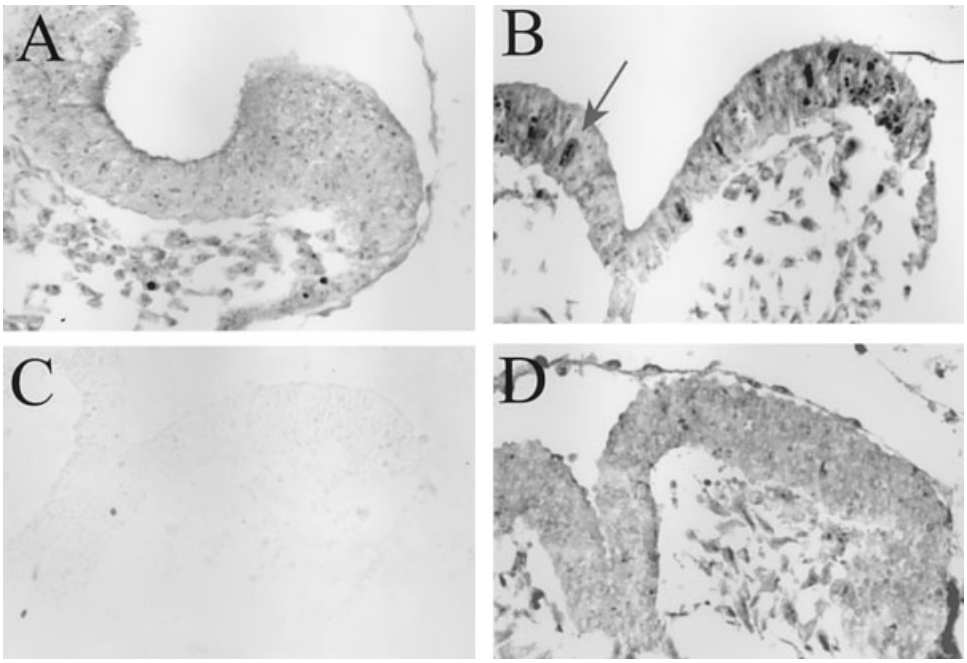


**Figure 2.** Attenuation of the cellular p53 response to 2CdA with PK11195 co-treatment. (**A**) Normal pattern of p53 immunoperoxidase staining in the headfold (optic pit region) on Day 8 of gestation. (**B**) Low-grade p53 protein response with specific nuclear labeling in the neuroepithelium (*arrow*) 4.5 hr after 5.0 mg/kg 2CdA exposure. (**C**) Negative control of 2CdA-treated embryo using normal serum in place of p53 antiserum. (**D**) Embryo with an attenuated p53 response collected 4.5 hr after 5.0 mg/kg 2CdA and 4.0 mg/kg PK11195 co-treatment.

$NADH/NAD^+$ ratio 1.5 hr postexposure and two-fold increase in the 16S rRNA signal relative to $\beta$-actin at 3.0 hr. Neither alteration was prevented when exposed embryos were co-treated with PK11195.

### Effect of Delayed PK11195 Administration

To localize the critical window for PK11195 intervention the co-treatment was delayed by 1.5 hr increments after 2CdA exposure. Each experimental group consisted of up to 6 litters and used 5.0 mg/kg 2CdA, which had a significant effect on malformation incidence but not fetal weight or resorption rate (Wubah et al., 2001). The ability of PK11195 to rescue fetuses decreased with time ($p \leq 0.05$, ANOVA). Post analysis with Dunnett's Multiple Comparison Test localized group differences (vs. control) to 2CdA alone and the group receiving a 6.0 hr delayed PK11195 administration (Fig. 4). The ability of PK11195 co-treatment to lower the risk of 2CdA-induced microphthalmia declined gradually with time-delay between 1.5–4.5 hr after 2CdA and then suddenly between 4.5–6.0 hr.

## DISCUSSION

The present study produced four new findings pertaining to the mechanism of action of 2CdA on CD-1 mouse embryos: 1) the teratogenic effect of 2CdA on early eye development can be blocked with PK11195, a small molecule that influences mitochondrial function; 2) the opportunity to rescue embryos with PK11195 lasted for approximately 4.5 hr after 2CdA exposure, thus partitioning the critical response period into revocable and irrevocable phases with respect to fixation of the pathogenic lesion; 3) metabolic reprogramming observed during the revocable phase (e.g., altered levels of NADH and 16S rRNA) was independent of PK11195 co-treatment; and 4) PK11195 downgraded the cellular p53 response induced with 2CdA by 4.5 hr post-exposure. We reported previously that 2CdA dosing scenarios that invoke low-grade p53 induction in the headfold had no demonstrable effect at 1.5 hr or 3.0 hr postexposure intervals, whereas dosing scenarios invoking high-grade induction had an early effect at 3.0 hr (Wubah et al., 2001). These findings link p53 protein induction between 3.0 and 4.5 hr with a critical event in the mechanism of teratogenesis and help build a coherent model in which the risk for 2CdA-induced microphthalmia correlated with inheritance of the wild-type *Trp53* allele (Wubah et al., 1996), the threshold dose for a p53 protein response (Wubah et al., 2001), and the fixed time interval leading to p53 protein induction (this study).

The two-fold drop in $NADH/NAD^+$ at 1.5 hr is the earliest cellular alteration so far observed in 2CdA treated embryos. Contrary to findings in leukemia cells where 2CdA activation of poly(ADP)ribose polymerase rapidly consumed $NAD^+$ pools (Carson et al., 1988), and counter to changes expected during hypoxia stimulation of anaerobic (glycolytic) metabolism (Obrosova et al., 2001), the fall in $NADH/NAD^+$ may reflect contamination of mitochondrial deoxynucleotide pools with 2CdA upon bioactivation with dGK-3. If so, then the effect to pyridine nucleotide balance can be attributed to increased activity of the electron transport chain that utilizes NADH as an electron donor or else to decreased activity of the Krebs tricarboxylic acid cycle that recycles NADH from $NAD^+$. The first scenario is consistent with the subsequent twofold rise in mitochondrial 16S rRNA levels because mtDNA genomic expression and mtRNA synthesis reflects the capacity for oxidative metabolism and specifically electron transport activity that is coupled to ATP levels (DasGupta et al., 2001). Therefore, we interpret these findings as an indication of metabolic reprogramming that the embryo reads from a signal to stimulate aerobic (oxidative) metabolism, a signal perhaps related to p53 protein function (Ibrahim et al., 1998; Donahue et al., 2001). Additional studies will be

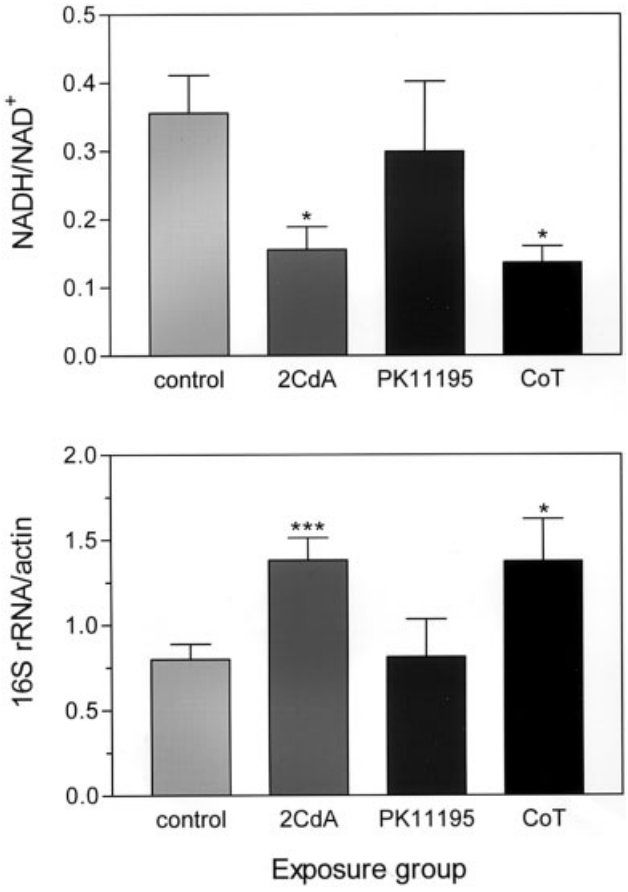


**Figure 3.** Lack of effect of PK11195 on early metabolic changes invoked with 2CdA on Day 8 of gestation. (**A**) Group means (±SE) for embryonic NADH/$NAD^+$ ratio 1.5 hr after 5.0 mg/kg 2CdA treatment on Day 8 with or without 4.0 mg/kg PK11195 co-treatment (CoT). ANOVA revealed significant differences in the group means ($p = 0.0153$); post-hoc analysis with Dunnett's multiple comparison test localized group differences ($^*p < 0.05$) to 2CdA and CoT groups relative to the control. Each group had at least five independent samples ($n \geq 5$). The effect on NADH was first detected at 1.0 hr and returned to normal by 2.0 hr (not shown). (**B**) Group means (±SE) for embryonic 16S rRNA/$\beta$-actin ratio 3.0 hr after 5.0 mg/kg 2CdA treatment on Day 8 with or without 4.0 mg/kg PK11195 (CoT). ANOVA revealed significant differences in the group means ($p = 0.0034$); post-hoc analysis with Dunnett's Multiple Comparison Test localized group differences ($^{***}p < 0.001$, $^*p < 0.05$) to 2CdA and CoT groups relative to the control. Each group had at least six independent samples ($n \geq 6$).

needed to test this hypothesis although it is clear that the putative signal induced with 2CdA is insensitive to PK11195 and hence upstream (or unrelated to) the locus of intervention.

The 16S rRNA response to 2CdA, as well as the failure of PK11195 to block this alteration, contrasts our own findings in two other systems (Donahue et al., 2001; O'Hara et al., 2002). In murine 3T3 cells, for example, the expression of 16S rRNA decreased in a PK11195-sensitive manner after transfection of cells with mitochondrial import competent, transdominant negative p53 miniprotein (Donahue et al., 2001). The other study in Day 9 mouse embryos observed acute downregulation of 16S rRNA in the prosencephalon after methylmercury exposure and again by a mechanism sensitive to PK11195 (O'Hara et al., 2002). To make sense of these complex effects more information is needed regarding the way that PK11195 acts on the mitochondrion to ameliorate teratogenesis.

## Does PK11195 Act Through the Peripheral Benzodiazepine Receptor (Bzrp)?

The *Bzrp* gene codes for an 18 kDa protein embedded in the outer mitochondrial membrane of many cell types (Garnier et al., 1994). It is designated a receptor by virtue of high-affinity binding to several classes of natural and synthetic ligands that can influence diverse physiological functions, including steroidogenesis, cell growth and death, and mitochondrial respiratory control (reviewed in Gavish et al., 1999). PK11195 has 7 nM binding affinity for this receptor and therefore is one of its most potent pharmacologic ligands (Verma et al., 1987).

The pharmacokinetics of PK11195 have been studied in various species including humans, rats, and mice (Le Fur et al., 1983; De Vos et al., 1999; Wala et al., 2000). Pretreating male CD-1 mice with an i.p. injection of 1.0 mg/kg PK11195 fully blocked binding activity of the Bzrp agonist Ro5-4864 in the brain, heart, and kidney; this effect was maximal within 30 min and persisted for 6–18 hr depending on the organ system (Le Fur et al., 1983). In female rats, an i.v. bolus of PK11195 (5–20 mg/kg in dimethylsulfoxide) is rapidly distributed between two compartments with elimination half-lives of 4.5–5.6 hr; plasma levels after a 5.0 mg/kg bolus gave a $C_{max}$ of 1630 ng/ml (~4500 nM) (Wala et al., 2000). Given that PK11195 has high affinity at the Bzrp and appears to have good pharmacokinetic properties in the mouse (De Vos et al., 1999), circulating levels of PK11195 in the mouse after a 4.0 mg/kg i.p. dose would be dramatically above the $K_i$ of the compound with respect to displacement of Bzrp ligands. Presently, we have no data suggesting what embryonic levels of PK11195 might occur after this dosage. Preliminary HPLC analysis with sensitivity of 14.6 pmol showed a small PK11195 peak in the maternal plasma at 20 min after 4.0 mg/kg i.p. injection but failed to reveal the characteristic peak in the fetus or placenta on Day 16 of gestation (not shown). Plasma or tissue protein binding could further impact the levels of "free" drug seen by the embryo. Direct exposure of early mouse embryos to PK11195 (5.0 $\mu$M in embryo culture) had similar effects as in vivo injection with respect to the ability of this ligand to counteract the effects of mercury poisoning on embryonic 16S rRNA expression (O'Hara et al., 2002). Although PK11195 is reasonably selective for binding to Bzrp and has effects consistent with direct action on the embryo, at concentrations >500 times the $K_i$ for Bzrp interaction it may have other activity as well that could confound the results of these experiments.

Bzrp transcripts were detected in the developing brain and its derivatives including the ocular primordium in developing rat embryos from as early as the headfold stage (Burgi et al., 1999). Evidence for the importance to normal early postimplantation development comes from efforts to generate a Bzrp knockout mouse, which proved embryonic lethal between Days 8–9 of gestation (Papadopoulos et al., 1997, personal communication with V. Papadopoulos). Furthermore, two strong candidate endogenous ligands of Bzrp, protoporphyrin IX (Taketani et al., 1995) and diaze-

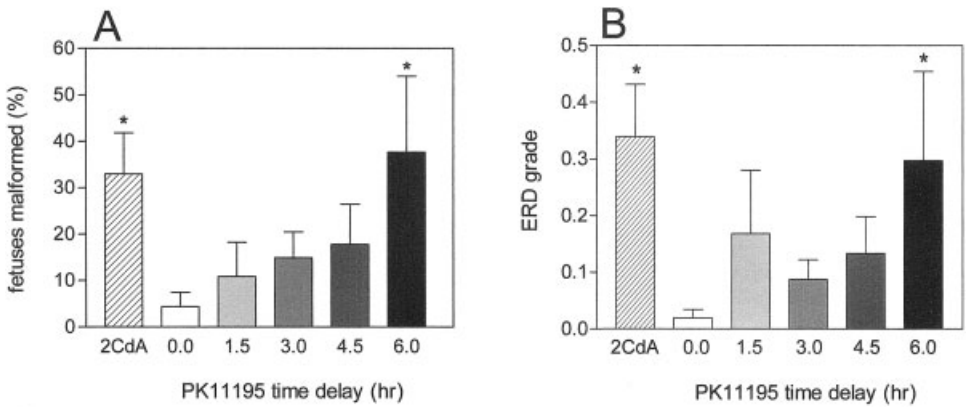


**Figure 4.** Time-dependent loss of rescue associated with delayed PK11195 administration. Malformation rate (*A*) and ERD grade (*B*) are shown as the litter mean ± SE for Day 17 of gestation after exposure to 2CdA and PK11195 co-treatment on Day 8. An independent 5.0 mg/kg 2CdA alone group ($n = 7$) is provided for reference. The time point designated at PK11195 time delay of 0.0 hr comes from the 7.5 mg/kg 2CdA and 4.0 mg/kg PK11195 group mentioned in Table 1 (4.4% malformations, ERD grade = 0.042). All other groups were treated with 2CdA and PK11195 with delayed co-treatment ($n = 3$ for 1.5 hr, $n = 6$ for all other groups). ANOVA revealed significant differences in the group means using control litter data from Table 1 ($p \leq 0.05$); post-hoc analysis with Dunnett's multiple comparison test localized group differences (*) to 2CdA alone and 6.0 hr co-treatment groups relative to the control group ($n = 7$).

pam binding inhibitor/acyl-CoA binding protein (Papadopoulos et al., 1997), were detected in neurulation-staged mouse and rat embryos (Burgi et al., 1999; O'Hara et al., 2002). These findings suggest that a potential Bzrp signaling axis is intact in the early embryo and that changes in the status or activity of this signaling axis occur during the critical period after 2CdA exposure, similar to what was observed in a recent study with methylmercury (MeHg) exposure on Day 9 of gestation (O'Hara et al., 2002).

We do not yet understand how exposure to 2CdA or MeHg alters Bzrp signaling. Clearly, the 16S rRNA response differed for these exposure scenarios with and without PK11195. For example, 5.0 mg/kg MeHg on Day 9 caused these transcripts to decrease significantly in the prosencephalon and the response was retracted with PK11195 co-treatment (O'Hara et al., 2002). In contrast, 5.0 mg/kg 2CdA on Day 8 caused these transcripts to increase in the headfold and the response was not retracted with PK11195. This discordance could reflect different toxicological mechanisms of action for 2CdA and MeHg or baseline changes in the status of Bzrp signaling axis as development advances from Day 8 to Day 9 of gestation. Isotherm studies of ligand-receptor interactions have led to the classification of PK11195 as an antagonist or partial agonist (Le Fur et al., 1983). Discordance for PK11195 with respect to the effects of 2CdA and MeHg on 16S rRNA expression can thus be explained by assuming that the status of Bzrp inversely couples with mitochondrial respiratory control (Anholt et al., 1985; Hirsch et al., 1988). PK11195 would function as a specific antagonist to endogenous Bzrp ligands elevated by MeHg (O'Hara et al., 2002) whereas 2CdA has the opposite effect to the intra-mitochondrial environment. We are presently investigating this possibility.

The capacity of PK11195 to intervene in the steps leading to nuclear accumulation of p53 protein suggests that PK11195 influences cellular activating signal for p53 protein induction, which itself may be a critical event leading to 2CdA-induced microphthalmia (Wubah et al., 1996, 2001). The nature of these signals is not clear; however, several possible roles reflect the localization of Bzrp to the outer mitochondrial membrane (Anholt et al., 1986; Dussossoy et al., 1996; see Casellas et al., 2002), including: negative control of aerobic (oxidative) metabolism (Anholt et al., 1985; Hirsch et al., 1988), positive regulation of cholesterol or heme biosynthesis (Taketani et al., 1995; Papadopoulos et al., 1997), protection of mitochondria from reactive oxygen species (Carayon et al., 1996; Wright and Reichenbecher, 1999), enhancement of organelle biogenesis (Shiraishi et al., 1991; Donahue et al., 2001), and apoptosis (Pastorino et al., 1996; Hirsch et al., 1998; Bono et al., 1999; Decaudin et al., 2002). Studies have shown that Bzrp antagonists (including PK11195) tend to be pro-apoptotic and receptor agonists are anti-apoptotic. In the context of 2CdA-induced microphthalmia pathogenesis has been directly correlated with p53-dependent apoptosis (Wubah et al., 1996). Thus, the protective effect of PK11195 on early eye development is consistent with its ability to suppress p53 protein induction and not the pro-apoptotic activity.

Elucidating the mechanisms through which teratogens perturb development requires an understanding of the progression of changes that link the direct effects of exposure with irrevocable dysmorphogenesis. Is there a window after exposure when the increased risk for malformation becomes irreversible and the subclinical pathological changes become fixed in time and space? For 2CdA the answer to this question appears to be "yes."

## ACKNOWLEDGMENTS

This publication was made possible by STAR grant R82744501-01 from the US Environmental Protection Agency (EPA), grant RO1 ES09120 from the National Institute of Environmental Health Sciences (NIEHS), and training grant T32 ES07282 from the NIEHS (R.J.D.). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views or policies of the NIEHS or the EPA.

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